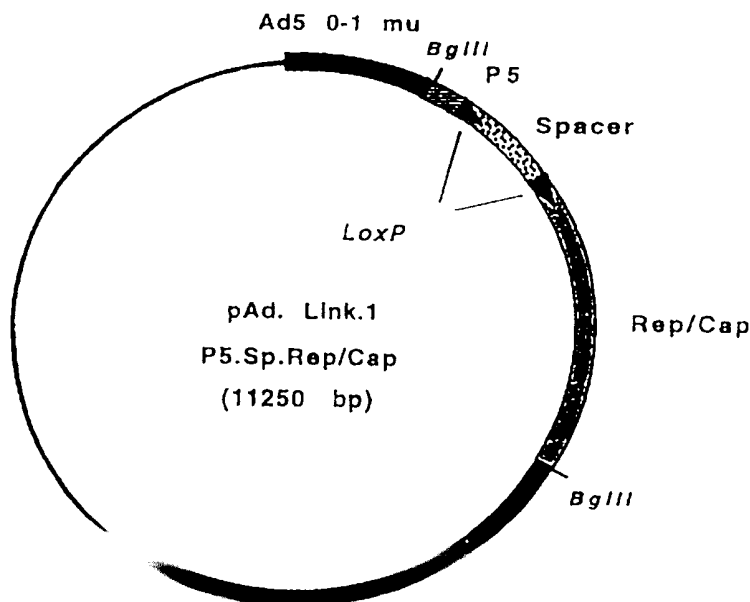


## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C12N 15/86, C07K 14/015, C12N 9/00, 9/52, 15/35, 7/01	A1	(11) International Publication Number: <b>WO 98/10086</b> (43) International Publication Date: 12 March 1998 (12.03.98)
(21) International Application Number: PCT/US97/15691 (22) International Filing Date: 4 September 1997 (04.09.97) (30) Priority Data: 60/025,323 6 September 1996 (06.09.96) US (60) Parent Application or Grant (63) Related by Continuation US 60/025,323 (CIP) Filed on 6 September 1996 (06.09.96) (71) Applicant (for all designated States except US): TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104-3147 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): WILSON, James, M. [US/US]; 1350 N. Avignon Drive, Gladwyne, PA 19035 (US). PHANEUF, Daniel [CA/US]; Apartment 1032, 834 Chestnut Street, Philadelphia, PA 19107 (US).	(74) Agents: KODROFF, Cathy, A. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	

(54) Title: METHODS USING CRE-LOX FOR PRODUCTION OF RECOMBINANT ADENO-ASSOCIATED VIRUSES



## (57) Abstract

Methods for efficient production of recombinant AAV are described. In one aspect, three vectors are introduced into a host cell. A first vector contains a Cre recombinase gene under the control of a Cre-inducible promoter. A second vector contains a Cre-inducible promoter. A third vector contains a Cre-inducible promoter. The Cre recombinase gene is expressed from the first vector and the Cre-inducible promoter is expressed from the second vector. The Cre recombinase gene is expressed from the third vector and the Cre-inducible promoter is expressed from the second vector. The Cre recombinase gene is expressed from the first vector and the Cre-inducible promoter is expressed from the third vector. The Cre recombinase gene is expressed from the second vector and the Cre-inducible promoter is expressed from the first vector. The Cre recombinase gene is expressed from the second vector and the Cre-inducible promoter is expressed from the third vector. The Cre recombinase gene is expressed from the third vector and the Cre-inducible promoter is expressed from the first vector. The Cre recombinase gene is expressed from the third vector and the Cre-inducible promoter is expressed from the second vector.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MY	Malaysia	US	United States of America
CA	Canada	IT	Italy				
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		

METHODS USING CRE-LOX FOR PRODUCTION OF  
RECOMBINANT ADENO-ASSOCIATED VIRUSES

Field of the Invention

This invention relates generally to production  
5 methods for recombinant viruses, and more specifically,  
to methods of producing recombinant adeno-associated  
viruses.

Background of the Invention

Adeno-associated virus (AAV) is a replication-  
10 deficient parvovirus, the genome of which is about 4.6 kb  
in length, including 145 nucleotide inverted terminal  
repeats (ITRs). Two open reading frames encode a series  
of *rep* and *cap* polypeptides. *Rep* polypeptides (*rep*78,  
*rep*68, *rep*62 and *rep*40) are involved in replication,  
15 rescue and integration of the AAV genome. The *cap*  
proteins (VP1, VP2 and VP3) form the virion capsid.  
Flanking the *rep* and *cap* open reading frames at the 5'  
and 3' ends are 145 bp inverted terminal repeats (ITRs),  
the first 125 bp of which are capable of forming Y- or T-  
20 shaped duplex structures. Of importance for the  
development of AAV vectors, the entire *rep* and *cap*  
domains can be excised and replaced with a therapeutic or  
reporter transgene [B. J. Carter, in "Handbook of  
Parvoviruses", ed., P. Tijsser, CRC Press, pp.155-168  
25 (1990)]. It has been shown that the ITRs represent the  
minimal sequence required for replication, rescue,  
packaging, and integration of the AAV genome.

When this nonpathogenic human virus infects a  
human cell, the viral genome integrates into chromosome  
30 19 resulting in latent infection of the cell. Production

of AAV is often achieved by co-infection of cells with  
virus such as adenovirus or herpesvirus. Upon infection  
with a helper virus, the AAV provirus is rescued and  
35 amplified, and both AAV and helper virus are produced.

The infecting parental ssDNA is expanded to duplex replicating form (RF) DNAs in a *rep* dependent manner. The rescued AAV genomes are packaged into preformed protein capsids (icosahedral symmetry approximately 20 nm in diameter) and released as infectious virions that have packaged either + or - ss DNA genomes following cell lysis.

AAV possesses unique features that make it attractive as a vector for delivering foreign DNA to cells. Various groups have studied the potential use of AAV in the treatment of disease states. Progress towards establishing AAV as a transducing vector for gene therapy has been slow for a variety of reasons. While the ability of AAV to integrate in quiescent cells is important in terms of long term expression of a potential transducing gene, the tendency of the integrated provirus to preferentially target only specific sites in chromosome 19 reduces its usefulness.

However, an obstacle to the use of AAV for delivery of DNA is lack of highly efficient schemes for encapsidation of recombinant genomes and production of infectious virions. See, R. Kotin, Hum. Gene Ther., 5:793-801 (1994)]. One such method involves transfecting the rAAV genome into host cells followed by co-infection with wild-type AAV and adenovirus. However, this method leads to unacceptably high levels of wild-type AAV. Incubation of cells with rAAV in the absence of contaminating wild-type AAV or helper adenovirus is associated with little recombinant gene expression. In the absence of *rep*, integration is inefficient and not

Producing AAV virions entails co-transfection with two different, yet complementing plasmids. One of these contains the therapeutic or reporter transgene sandwiched

between the two *cis* acting AAV ITRs. The AAV components that are needed for rescue and subsequent packaging of progeny recombinant genomes are provided in *trans* by a second plasmid encoding the viral open reading frames for *rep* and *cap* proteins. Overexpression of Rep proteins have some inhibitory effects on adenovirus and cell growth [J. Li et al, *J. Virol.*, 71:5236-5243 (1997)]. This toxicity has been the major source of difficulty in providing these genes in *trans* for the construction of a useful rAAV gene therapy vector.

There remains a need in the art for the methods permitting the efficient production of AAV and recombinant AAV viruses for use as vectors for somatic gene therapy.

#### Summary of the Invention

The present invention provides methods which permit efficient production of rAAV, which overcome the difficulties faced by the prior art. This method is particularly desirable for production of recombinant AAV vectors useful in gene therapy. The method involves providing a host cell with

- (a) a *cre* transgene, which permits splicing out of the *rep* and *cap* gene inhibitory sequences that when removed lead to activation of *rep* and *cap*;
- (b) the AAV *rep* and *cap* genes, 5' to these genes is a spacer which is flanked by lox sites;
- (c) a minigene comprising a therapeutic transgene flanked by AAV inverse terminal repeats (ITRs); and

The present invention provides a method for producing a rAAV which comprises introducing into a host cell a first vector containing the *cre*

transgene under regulatory control of sequences which express the gene product thereof *in vitro*, a second vector containing a spacer flanked by lox sites, which is 5' to the rep and cap genes, and a third vector  
5 containing a therapeutic transgene flanked by AAV ITRs. These vectors may be plasmids or recombinant viruses. One of the vectors can be a recombinant adenovirus or herpesvirus, which can provide to the host cell the essential viral helper functions to produce a rAAV  
10 particle. However, if all the vectors are plasmids, the cell must also be infected with the desired helper virus. The cell is then cultured under conditions permitting production of the cre recombinase. The recombinase causes deletion of the spacer flanked by lox sites  
15 upstream of the rep/cap genes. Removal of the spacer allows the rep and cap genes to be expressed, which in turn allows packaging of the therapeutic transgene flanked by AAV ITRs. The rAAV is harvested thereafter.

In another aspect, the invention provides a  
20 method wherein a host cell expressing cre recombinase is co-transfected with a vector carrying a spacer flanked by lox sites 5' to the rep and cap genes, and a vector containing the therapeutic minigene above. With the provision of helper functions by a means described  
25 herein, the cell is then cultured under appropriate conditions. When cultured, the cre recombinase causes deletion of the spacer thus activating expression of rep/cap, resulting in the rAAV as described above.

In yet another aspect, the present invention  
30 provides rAAV vectors produced by the methods of the

and are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 is a schematic illustration of a 1600 bp DNA fragment containing green fluorescent protein (GFP) cDNA, an intron and a polyadenylation (pA or polyA) signal useful as a spacer in a vector of the invention.

Fig. 2 is a schematic illustration of a 1000 bp DNA fragment containing the gene encoding neomycin resistance ( $neo^R$ ) and a polyA useful as a spacer.

Fig. 3 illustrates a plasmid pG.CMV.nls.CRE, useful for transfection of human embryonic kidney 293 cells in the method of the invention.

Fig. 4 illustrates a plasmid pAd.P5.Sp.Rep/Cap, useful in the method of the invention.

Fig. 5 illustrates the construction of the recombinant adenovirus, Ad.CMV.NLS-CRE, useful in the method of the invention.

Fig. 6A illustrates the structure of the Ad.CAG.Sp.LacZ virus.

Fig. 6B provides the Southern blot analysis of genomic DNA isolated from 293 cells infected with the LacZ virus at a m.o.i. of 1 and cut with *NotI*. The 1000 bp  $^{32}P$ -NEO spacer was used as a probe. After the digestion with *NotI* a 6200 bp restriction fragment (without cre-mediated recombination) and/or a 5200 bp restriction fragment (with cre-mediated recombination) can be detected.

Fig. 6C provides the Southern blot analysis of genomic DNA isolated from 293 cells infected with the LacZ virus at a m.o.i. of 10 and cut with *NotI*. The 1000 bp  $^{32}P$ -NEO spacer was used as a probe. After the

restriction fragment (without cre-mediated recombination), can be detected.

Fig. 6D provides the Southern blot analysis genomic DNA isolated from 293 cells infected with the LacZ virus at a m.o.i. of 100 and cut with *NotI*. The 1000 bp <sup>32</sup>P-NEO spacer was used as a probe. After the  
5 digestion with *NotI* a 6200 bp restriction fragment (without cre-mediated recombination) and/or a 5200 bp restriction fragment (with cre-mediated recombination) can be detected.

Fig. 7 illustrates the structure of the  
10 Ad.Tre.CMV.GFP.Rep/Cap virus.

#### Detailed Description of the Invention

The invention provides a method for rAAV production using the cre-lox system, which overcomes the difficulties previously experienced in providing  
15 efficient production systems for recombinant AAV. The method of this invention produces rAAV carrying therapeutic transgenes, which are particularly useful in gene therapy applications.

In summary, the method involves culturing a  
20 selected host cell which contains  
(a) a cre transgene  
(b) the AAV rep and cap genes, 5' to these genes is a spacer flanked by lox sites;  
(c) a minigene comprising a therapeutic  
25 transgene flanked by AAV ITRs; and  
(d) adenovirus or herpesvirus helper functions.

The use of the term "vector" throughout this specification refers to either plasmid or viral vectors,

wherein "cell" means any mammalian cell which is capable of functioning as an adenovirus packaging cell, i.e., expresses any adenovirus proteins essential to the



production of AAV, such as HEK 293 cells and other packaging cells. By the term "minigene" is meant the sequences providing a therapeutic transgene in operative association with regulatory sequences directing  
5 expression thereof in the host cell and flanked by AAV ITRs. The term "transgene" means a heterologous gene inserted into a vector.

Desirably, components (a), (b) and (c) may be carried on separate plasmid sequences, or carried as a  
10 transgene in a recombinant virus. Alternatively, the cre protein may be expressed by the selected host cell, therefor not requiring transfection by a vector. For each of these components, recombinant adenoviruses are currently preferred. However, using the information  
15 provided herein and known techniques, one of skill in the art could readily construct a different recombinant virus (i.e., non-adenovirus) or a plasmid vector which is capable of driving expression of the selected component in the host cell. For example, although less preferred  
20 because of their inability to infect non-dividing cells, vectors carrying the required elements of this system, e.g., the cre recombinase, may be readily constructed using e.g., retroviruses or baculoviruses. Therefore, this invention is not limited by the virus or plasmid  
25 selected for purposes of introducing the cre recombinase, rep/cap, or minigene into the host cell.

Desirably, however, at least one of the vectors is a recombinant virus which also supplies the helper functions (d) to the cell. Alternatively, the helper  
30 functions may be supplied by co-infecting the cell with a

### A. The Cre Transgene

The cre protein is a recombinase isolated from bacteriophage P1 which recognizes a specific sequence of 34 bp (*loxP*). Recombination between two *loxP* sites (catalyzed by the cre protein) causes, in certain cases, the loss of sequences flanked by these sites [for a review see N. Kilby et al, Trends Genet., 9:413-421 (1993)]. The sequences of cre are provided in N. Sternberg et al, J. Mol. Biol., 187:197-212 (1986) and may alternatively be obtained from other commercial and academic sources. The expression of the cre protein in the cell is essential to the method of this invention.

Without wishing to be bound by theory, the inventors believe that the expression of cre recombinase in the host cell permits the deletion of the "spacer" DNA sequence residing between the promoter and rep/cap genes in the second vector. This deletion of rep and cap gene inhibitory sequences, allows expression and activation of the rep and cap proteins and resulting in the replication and packaging of the AAV genome.

The cre protein may be provided in two alternative ways. The gene encoding the protein may be a separate component transfected into the desired host cell. Alternatively, the host cell selected for expression of the rAAV may express the cre protein constitutively or under an inducible promoter.

### B. Triple Infection/Transfection Method

In one embodiment of the present invention, the method employs three vectors, i.e., recombinant viruses or plasmids, to infect/transfect a

...sequences. A second vector comprises the AAV rep and cap genes downstream of a spacer sequence which is flanked by lox sites and which itself is

downstream of expression control sequences. A third vector comprises the therapeutic minigene, i.e., a transgene flanked by AAV ITRs and regulatory sequences. Suitable techniques for introducing these vectors into the host cell are discussed below and are known to those of skill in the art. When all vectors are present in a cell and the cell is provided with helper functions, the rAAV is efficiently produced.

#### 1. First Vector

As stated above, in a preferred embodiment, a first vector is a recombinant replication-defective adenovirus containing the cre transgene operatively linked to expression control sequences in the site of adenovirus E1 deletion, e.g., Ad.CMV.NLS-CRE. See Fig. 5. Preferably, as in the examples below, the cre gene is operably linked to a suitable nuclear localization signal (NLS). A suitable NLS is a short sequence, i.e., in the range of about 21 bp, and may be readily synthesized using conventional techniques, or engineered onto the vector by including the NLS sequences in a PCR primer. As described in detail in Example 1 below, the cre gene and a nuclear localization signal (NLS) are obtained from a previously described plasmid.

Desirably, the cre gene is under the control of a cytomegalovirus (CMV) immediate early promoter/enhancer [see, e.g., Boshart et al, Cell, 41:521-530 (1985)]. However, other suitable promoters may be readily selected by one of skill in the art. Useful promoters may be constitutive promoters or regulated (inducible) promoters, which will enable

control of the expression of the cre gene. Without limitation, the Rous sarcoma virus LTR promoter/enhancer. Still other promoter/enhancer sequences may be selected by one of skill in the art.

In addition, the recombinant virus also includes conventional regulatory elements necessary to drive expression of the cre recombinase in a cell transfected with the vector. Such regulatory elements are known to those of skill in the art, including without limitation, polyA sequences, origins of replication, etc.

## 2. Second Vector

Another, "second", vector useful in this embodiment of the method is described in Example 2 as Ad.sp.Rep/Cap. It contains the AAV rep and cap genes downstream of a spacer sequence which is flanked by lox sites and which itself is downstream of expression control sequences.

The AAV rep and cap sequences are obtained by conventional means. Preferably, the promoter is the AAV P5 promoter. However, one of skill in the art may readily substitute other suitable promoters. Examples of such promoters are discussed above in connection with the first vector.

The spacer is an intervening DNA sequence (STOP) between the promoter and the gene. It is flanked by loxP sites and contains multiple translational start and stop codons. The spacer is designed to permit use of a "Recombination-Activated Gene Expression (RAGE)" strategy [B. Sauer, Methods Enzymol., 225:890-900 (1993)]. Such a strategy controls the expression of a given gene (in this case, rep/cap). The spacer must be excised by expression of the cre protein of the first vector and its interaction with the lox sequences to express rep/cap.

Fragment containing the GFP cDNA, an intron and a polyadenylation signal (Fig. 1) which was derived from a

commercial plasmid (Clontech) as described below. A second preferred spacer is a 1300 bp fragment containing translational start and stop sequences obtained as a 1.3 kbp ScaI-SmaI fragment of pBS64 as described [M. Anton and F. Graham, J. Virol., 69:4600-4606 (1995)]. Another desirable spacer is a 1000 bp fragment containing the neomycin resistance coding sequence and a polyadenylation signal [Y. Kanegae et al, Nucl. Acids Res., 23:3816-3821 (1995)] (see, Fig. 2).

Using the information provided herein, one of skill in the art may select and design other suitable spacers, taking into consideration such factors as length, the presence of at least one set of translational start and stop signals, and optionally, the presence of polyadenylation sites. These spacers may contain genes, which typically incorporate the latter two elements (i.e., the start/stop and polyA sites). Desirably, to reduce the possibility of recombination, the spacer is less than 2 kbp in length. However, the invention is not so limited.

As stated above, the spacer is flanked by *loxP* sites, which are recognized by the cre protein and participate in the deletion of the spacer. The sequences of *loxP* are publicly available from a variety of sources [R. H. Hoess and K. Abremski, Proc. Natl. Acad. Sci., 81: 1026-1029 (1984)]. Upon selection of a suitable spacer and making use of known techniques, one can readily engineer *loxP* sites onto the ends of the spacer sequence for use in the method of the invention.

In addition, the recombinant virus

with the recombinant virus, following excision of the

loxP-flanked spacer by the cre recombinase. Such regulatory elements are known to those of skill in the art.

### 3. Third Vector

5 The third vector contains a minigene, which is defined as a sequence which comprises a suitable transgene, a promoter, and other regulatory elements necessary for expression of the transgene, all flanked by AAV ITRs. In the examples below, where the third vector  
10 carries the LacZ gene, the presence of rAAV is detected by assays for beta-galactosidase activity. However, desirably, the third vector carries a therapeutic gene which can be delivered to an animal via the rAAV produced by this method.

15 The AAV sequences employed are preferably the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences [See, e.g., B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp.155-168 (1990)]. The ITR sequences are about 143 bp  
20 in length. Preferably, substantially the entire sequences encoding the ITRs are used in the vectors, although some degree of minor modification of these sequences is expected to be permissible for this use. The ability to modify these ITR sequences is within the  
25 skill of the art. [See, e.g., texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual.", 2d edit., Cold Spring Harbor Laboratory, New York (1989); Carter et al, cited above; and K. Fisher et al., J. Virol., 70:520-532 (1996)].

30 The AAV ITR sequences may be obtained

employed in the construction of this invention. The selection of the AAV is not anticipated

to limit the following invention. A variety of AAV strains, types 1-4, are available from the American Type Culture Collection or available by request from a variety of commercial and institutional sources. In the  
5 following exemplary embodiment an AAV-2 is used for convenience.

The 5' and 3' AAV ITR sequences flank the selected transgene sequence and associated regulatory elements. The transgene sequence of the vector is a  
10 nucleic acid sequence heterologous to the AAV sequence, which encodes a polypeptide or protein of interest. The composition of the transgene sequence will depend upon the use to which the resulting vector will be put. For example, one type of transgene sequence includes a  
15 reporter sequence, which upon expression produces a detectable signal. Such reporter sequences include without limitation an *E. coli* beta-galactosidase (*LacZ*) cDNA, an alkaline phosphatase gene and a green fluorescent protein gene. These sequences, when  
20 associated with regulatory elements which drive their expression, provide signals detectable by conventional means, e.g., ultraviolet wavelength absorbance, visible color change, etc.

A more preferred type of transgene  
25 sequence is a therapeutic gene which expresses a desired gene product in a host cell. These therapeutic nucleic acid sequences typically encode products for administration and expression in a patient *in vivo* or *ex vivo* to replace or correct an inherited or non-inherited  
30 genetic defect or treat an epigenetic disorder or

In addition to the major elements identified above, the minigene also includes conventional  
35 regulatory elements necessary to drive expression of the

transgene in a cell transfected with this vector. Thus, the minigene comprises a selected promoter which is linked to the transgene and located within the transgene between the AAV ITR sequences.

5                    Selection of the promoter used to drive expression of the transgene is a routine matter and is not a limitation of the vector. Useful promoters include those which are discussed above in connection with the first vector component.

10                   The minigene also desirably contains heterologous nucleic acid sequences including sequences providing signals required for efficient polyadenylation of the transcript and introns with functional splice donor and acceptor sites. A common poly-A sequence which  
15 is employed in the exemplary vectors of this invention is that derived from the papovavirus SV-40. The poly-A sequence generally is inserted following the transgene sequences and before the 3' AAV ITR sequence. A common intron sequence is also derived from SV-40, and is  
20 referred to as the SV-40 T intron sequence. A minigene of the present invention may also contain such an intron, desirably located between the promoter/enhancer sequence and the transgene. Selection of these and other common vector elements are conventional and many such sequences  
25 are available [see, e.g., Sambrook et al, and references cited therein].

                  The rAAV vector containing the minigene may be carried on a plasmid backbone and used to transfect a selected host cell or may be flanked by viral  
30 sequences (e.g., adenoviral sequences) which permit it to

known techniques. See, e.g., International patent applications WO96/13598, published May 9, 1996;



WO95/23867 published Sept. 8, 1995, and WO 95/06743 published March 9, 1995, which are incorporated by reference herein.

5                   C.   *Host Cell/Double Infection or Transfection System*

                  In another embodiment of the method of this invention, a packaging cell line is constructed which expresses the cre recombinase. According to this aspect of the method, this cell line expressing the cre  
10 recombinase can be substituted for the vector or plasmid bearing the cre gene, as described above. Thus, only the second and third vectors described above are subsequently introduced into the cell.

                  An exemplary suitable cre expressing cell  
15 line has been generated using the vector illustrated in Fig. 3. Generation of this cell line is described in detail in Example 4 below. However, the present invention is not limited to these constructs. Given the information provided herein, one of skill in the art can  
20 readily generate another plasmid containing a suitable selectable marker (e.g., neo<sup>R</sup>). Such a plasmid may then be used for the generation of a cre recombinase-expressing cell line according to the invention.

                  Having obtained such a cre-expressing cell  
25 line, this cell line can be infected (or transfected) with the vector containing the rep/cap genes and the vector containing the minigene described above.

                  D.   *Production of Vectors and rAAV*

                  Assembly of the selected DNA sequences  
30 contained within each of the vectors described above

as illustrated above, use of overlapping oligonucleotide sequences of the adenovirus, AAV genome combined with

polymerase chain reaction, and any other suitable methods which provide the desired nucleotide sequence.

Whether using the three vector system, or the cre-expressing host cell and two vectors, introduction of the vectors into the host cell is accomplished using known techniques. Where appropriate, standard transfection and co-transfection techniques are employed, e.g.,  $\text{CaPO}_4$  transfection techniques using the complementation human embryonic kidney (HEK) 293 cell line (a human kidney cell line containing a functional adenovirus Ela gene which provides a transacting Ela protein). Other conventional methods employed in this invention include homologous recombination of the viral genomes, plaquing of viruses in agar overlay, methods of measuring signal generation, and the like.

Following infection/transfection, the host cell is then cultured under standard conditions, to enable production of the rAAV. See, e.g., F. L. Graham and L. Prevec, Methods Mol. Biol., 7:109-128 (1991). Desirably, once the rAAV is identified by conventional means, it may be recovered using standard techniques and purified.

The following examples illustrate the preferred methods of the invention. These examples are illustrative only and are not intended to limit the scope of the invention.

#### Example 1 - Construction of Ad.CMV.NLS-CRE

The construction of a recombinant adenovirus containing a nuclear localization signal and the cre gene

The nls-Cre cDNA was isolated from the plasmid pexCANCRE [Y. Kanegae et al, Nucl. Acids Res., 23:3816-3821 (1995)] by digestion with SfiI and PstI and then

blunt ended with Klenow and T4 DNA polymerase. The NLS-Cre fragment was then cloned into the EcoRV site of the plasmid pAd.CMV.Link (a plasmid containing the human Ad5 sequences, map units 0 to 16, which is deleted of E1a and E1b as described in X. Ye et al, J. Biol. Chem., 271:3639-3646 (1996). The orientation and presence of the nuclear localization signal in the resulting plasmid pAd.CMV.NLS-CRE was verified by sequencing.

To produce the recombinant adenovirus carrying the cre transgene, the pAd.CMV.NLS-CRE recombinant vector was co-transfected with the Ad dl327 backbone into 293 cells. Ten days later, 15 plaques were picked up and 5 of them were expanded on 293 cells. Viruses were screened for their recombinase activity by assessing their ability to remove a spacer positioned between the CAG promoter (beta-actin) and the bacterial LacZ coding sequence using an adenoviral construct described in Y. Kanegae et al, Nucl. Acids Res., 23:3816-3821 (1995). Two viruses tested positive for beta-galactosidase activity, indicating cre recombinase activity. As desired, these recombinant viruses may be purified by two rounds of plaque purification.

#### Example 2 - Construction of Ad.sp.Rep/Cap

An exemplary recombinant adenovirus containing the AAV rep and cap genes may be produced as follows.

An AAV P5 promoter was obtained from the 121 bp XbaI-BamHI fragment from plasmid psub201, which contains the entire AAV2 genome [R.J. Samulski et al, J. Virol., 61:3096-3101 (1987)] by PCR using the following primer

5'-TACATGGAGGGGCTGGAGTCCGTGA-

BamP5 rightward: SEQ ID NO:3:

GGCCGGATCCAACGCGCAGCCGCCATGCCG;

Bam P5 leftward: SEQ ID NO:4:

GGCCGGATCCCAAACCTCCCGCTTCAAAAT;

SacI leftward: SEQ ID NO:5:

GGCCGAGCTCAGGCTGGGTTTTGGGGAGCA.

5           A 5' portion of the Rep/Cap gene was similarly excised via PCR from a BamI-SacI fragment (504 bp) obtained from psub201. The BamHI PCR primer creates a unique site between the rep mRNA and the first rep ATG.

10           The P5 promoter and the Rep/Cap gene fragment were subcloned into the XbaI-SacI sites of the pSP72 vector (Promega), resulting in P5.Rep/Cap. The spacer DNA, a 1300 bp fragment flanked by loxP sites, was obtained from the plasmid pMA19 [M. Anton and F. Graham, J. Virol., 69:4600-4606 (1995)] following digestion with  
15   BamHI. This spacer DNA was cloned into the unique BamHI site of the P5.Rep/Cap construct, resulting in the P5.Spacer.Rep/Cap construct.

20           The complete fragment containing the P5 promoter, the spacer and the rep/cap genes was obtained by subcloning the 3' portion of the Rep/Cap gene (SacI/blunt ended fragment, 3680 bp) into the SacI-EcoRV sites of the P5.Spacer.Rep/Cap plasmid. The 3' portion of the Rep/cap gene was isolated from the SSV9 plasmid (which contains a complete wild-type AAV genome) as a  
25   SacI-blunt ended fragment. This involved digesting SSV9 with XbaI, filling the XbaI site with Klenow and liberating the fragment by digesting with SacI.

30           The complete fragment containing the P5 promoter, the spacer and the rep/cap sequence was subcloned into the BglIII site of the pAd.link vector.

The BglIII site located at the 3' end of the multiple cloning site of pSP72.

The resulting plasmid (11250 bp) contains Ad5 map units (mu) 0-1, the P5 promoter, the spacer sequence flanked by loxP sites, rep/cap, and Ad5 mu 9-16. This plasmid is termed pAd.P5.spacer.Rep/Cap [Fig. 4].

5           To produce recombinant adenovirus capable of expressing rep and cap, pAd.P5.spacer.Rep/Cap was first used to transform a cre-expressing bacterial strain *E. coli* strain BNN132 (ATCC Accession No. 47059) in order to determine whether the spacer could be removed after  
10 recombination between the loxP sites (catalyzed by the cre recombinase). Analysis on agarose gels of the plasmid DNA isolated from several transformed colonies showed that, indeed, most of the constructs analyzed lost the spacer following transformation (data not shown).

15           The plasmid P5.spacer.Rep/Cap was also co-transfected with the Ad dl327 backbone in HEK 293 cells. Ten days later, 20 plaques were picked up and expanded. The structure of the viruses was analyzed by Southern blot using the complete AAV genome and the 1300 bp DNA  
20 spacer as probes. One plaque (P3) showed the expected band pattern after digestion with the restriction enzyme BamHI (data not shown).

          Similar constructs may be made using other suitable spacers. For example, a 1600 bp spacer was  
25 derived from plasmid pHGFP-S65T plasmid (Clontech) which contains the humanized GFP gene. pHGFP-S65T was cut with the restriction enzymes HindIII and BamHI. After adding a BglII linker at the 5' end (BglII is compatible with BamHI), the 1.6 kb fragment was subcloned into the BamHI  
30 site of the flox vector [H. Gu et al, Science, 265:103-

and subsequent insertion into the EcoRV site of the Bluescript II cloning vector

(Stratagene). The resulting GFP spacer can be used to construct a P5.spacer.Rep/cap plasmid or adenovirus as described above.

### Example 3 - Production of rAAV

5                   The supernatant from several plaques  
(containing viruses) obtained from the study described in  
Example 2 was tested for the ability to produce AAV in a  
functional assay involving the adenovirus encoding the  
cre protein constructed as described in Example 1 above  
10                   and pAV.CMVLacZ.

The plasmid AV.CMVLacZ is a rAAV cassette in  
which rep and cap genes are replaced with a minigene  
expressing  $\beta$ -galactosidase from a CMV promoter. The  
linear arrangement of AV.CMVLacZ includes:

- 15                   (a) the 5' AAV ITR (bp 1-173) obtained by PCR  
using pAV2 [C. A. Laughlin et al, Gene, 23: 65-73 (1983)]  
as template [nucleotide numbers 365-538 of SEQ ID NO:1];  
                  (b) a CMV immediate early enhancer/promoter  
[Boshart et al, Cell, 41:521-530 (1985); nucleotide  
20                   numbers 563-1157 of SEQ ID NO:1],  
                  (c) an SV40 intron (nucleotide numbers 1178-  
1179 of SEQ ID NO:1),  
                  (d) *E. coli* beta-galactosidase cDNA  
(nucleotide numbers 1356 - 4827 of SEQ ID NO:1),  
25                   (e) an SV40 polyadenylation signal (a 237  
BamHI-BclI restriction fragment containing the  
cleavage/poly-A signals from both the early and late  
transcription units; nucleotide numbers 4839 - 5037 of  
SEQ ID NO:1) and

EXAMPLE 4

The functional assay was performed by infecting  
293 cells with the cre virus and the Rep/Cap virus

(multiplicity of infection (MOI) 10) followed by a transfection 2 hours later with 5  $\mu$ g pAV.CMVLacZ. Forty-eight hours later, cells were harvested and freeze-thawed. One-fifth of the supernatant (containing rAAV) was used to infect 293 cells. Twenty-four hours later an X-gal assay was performed.

Viruses from plaque #3 yielded positive for beta-galactosidase transduction in this assay. Supernatant from plaque #3 was used in a second round of purification (plaque amplification). Twenty plaques were picked up and expanded.

#### Example 4 - Production of Cre Expressing Cell Line

A plasmid vector, pG.CMV.nls.cre was constructed as follows for use in transfecting 293 cells. The nls-Cre cDNA was isolated from the plasmid pexCANCRE (Kanegae, cited above) as described in Example 1 above. The nls-Cre fragment was then subcloned into the XbaI sites of vector pG downstream of a CMV promoter. This plasmid vector is illustrated in Figure 3 and contains a human growth hormone (hGH) termination sequence, an SV40 ori signal, a neomycin resistance marker, an SV40 polyadenylation site, an ampicillin marker, on a backbone of pUC19.

This plasmid was transfected into 293 cells using conventional techniques. Cells were selected in the presence of G-418 for neomycin resistance. Cells were identified by infecting them at different MOI (1 to 100) with Ad.CAG.Sp.LacZ, an adenovirus containing the bacterial LacZ coding sequence separated from its beta-

When infected, the basic adenovirus will remove the spacer fragment inducing the expression of the LacZ gene. After X-gal staining, six cell lines were

found to be positive. DNA from these infected cells was isolated and analyzed by Southern blot using the spacer DNA (NEO) as a probe. Results shown in Fig. 6A, with reference to Table 1, and Figs. 6B - 6D indicate that cell line #2 can remove the DNA spacer with much more efficacy than the other 293/cre cell lines analyzed.

Table 1

<u>NEO Probe</u>	
<u>Without Recombination</u>	<u>With Recombination</u>
6200	6200
	5200

#### Example 5 - Generation of the Ad.GFP Rep/Cap

As described in Example 2 for the construction of the Ad.Sp.Rep/Cap virus, the link plasmid containing the P5 promoter, the GFP spacer flanked by two loxP sites and the Rep and Cap coding sequences was co-transfected with the Ad dl327 backbone into HEK 293 cells. Ten days later, 20 plaques were picked up and expanded. During this expansion, the monolayer of HEK 293 cells were screened for the expression of GFP by microscopic analysis using a mercury lamp with a 470-490 nm band-pass excitation filter (Nikon). One of the monolayers (from plaque #13) showed a region positive for the expression of GFP. This region was further expanded and purified by two other rounds of plaque purification. The presence of the Ad.GFP.Rep/Cap virus was monitored by the expression

of GFP. Antibodies: AMERICAN RESEARCH PRODUCTS

One cell lysate (from one purified plaque) containing the Ad.GFP rep/cap was used in order to infect 293 cells



(adenovirus preparation with 40 x 150 mm dishes of HEK 293 cells). A total of  $6.86 \times 10^{13}$  particles/ml were obtained after purification. This virus is currently being tested for the production of rAAV, as described in Example 3.

Example 6 - Construction of the Ad.TRE.CMV.GFP.Rep/Cap

Fig. 7 shows the final structure of the Ad.TRE.CMV.Rep/Cap virus. The AAV P5 promoter was replaced by the tetracycline (Tet) inducible promoter (Clontech). This promoter contains the tetracycline responsive elements (TRE) followed by the CMV minimal promoter without the CMV enhancer. This promoter is inducible in the presence of the antibiotic doxycycline (Sigma) in the 293/Tet-On cell line (Clontech) which contains a stable gene expressing the rTetR (reverse Tet repressor) fused to the GP16 transcriptional activation domain. The objective here is to construct a double inducible expression system in order to limit the expression of the cytotoxic Rep gene products. In order to fully induce the expression of the Rep and Cap genes, the virus must be in the presence of 1- the cre recombinase (in order to delete the GFP spacer as described previously) and 2- the Tet-On inducible factor doxycycline (DOX).

The link plasmid containing the construct described above was used to transfect HEK 293 cells in the presence or the absence of DOX and/or the cre recombinase (from the adenovirus expressing nls-cre). Proteins from cell homogenates were analyzed by Western

DESCRIPTION

In order to construct pAd.TRE.CMV.link.1, the pTRE plasmid (Clontech) was cut with the restriction

endonucleases *Xho* and *EcoRI* to isolate the TRE and the minimal CMV promoter. The *Xho* and *EcoRI* sites were filled with Klenow and the 448 bp fragment was inserted into the *EcoRV* site of the pAdlink.1 plasmid. The  
5 GFP.Rep/Cap fragment was subsequently cut with *ClaI* and *BglIII* and inserted into the pAd.TRE.CMV.link.1 cut with *ClaI* and *BamHI*.

This link recombinant plasmid was co-transfected with the Ad dl327 backbone in HEK 293 cells.  
10 Ten days later, 20 plaques were picked up and expanded. These plaques are currently being analyzed for the expression of GFP and the Rep and Cap proteins. Two adenoviruses expressing large amounts of rep proteins were identified. These viruses are currently being  
15 purified and studied.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to  
20 the processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Trustees of the University of Pennsylvania  
Wilson, James M.  
Phaneuf, Daniel
- (ii) TITLE OF INVENTION: Methods using Cre-Lox for Production of  
Recombinant Adeno-Associated Viruses
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Howson and Howson
  - (B) STREET: Spring House Corporate Cntr, PO Box 457
  - (C) CITY: Spring House
  - (D) STATE: Pennsylvania
  - (E) COUNTRY: USA
  - (F) ZIP: 19477
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: WO
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 60/025,323
  - (B) FILING DATE: 06-SEP-1996
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Kodroff, Cathy A.
  - (B) REGISTRATION NUMBER: 33,980
  - (C) REFERENCE/DOCKET NUMBER: GNVPN.024CIP1PCT
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 215-540-9200
  - (B) TELEFAX: 215-540-5818

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 10398 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: unknown

GAATTCGCTA GCATCATCAA TAATATACCT TATTTTGGAT TGAAGCCAAT ATGATAATGA 60

GGGGGTGGAG TTTGTGACGT GGC GCGGGGCG GTGGGAACGG GCGGGGTGAC GTAGTAGTGT 120

26

GGCGGAAGTG	TGATGTTGCA	AGTGTGGCGG	AACACATGTA	AGCGACGGAT	GTGGCAAAAG	180
TGACGTTTTT	GGTGTGCGCC	GGTGTACACA	GGAAGTGACA	ATTTTCGCGC	GGTTTTAGGC	240
GGATGTTGTA	GTAAATTTGG	GCGTAACCGA	GTAAGATTTG	GCCATTTTCG	CGGGAAAAC	300
GAATAAGAGG	AAGTGAAATC	TGAATAATTT	TGTGTTACTC	ATAGCGCGTA	ATATTTGTCT	360
AGGGAGATCT	GCTGCGCGCT	CGCTCGCTCA	CTGAGGCCGC	CCGGGCAAAG	CCCGGGCGTC	420
GGGCGACCTT	TGGTCGCCCC	GCCTCAGTGA	GCGAGCGAGC	GCGCAGAGAG	GGAGTGGCCA	480
ACTCCATCAC	TAGGGGTTCC	TTGTAGTTAA	TGATTAACCC	GCCATGCTAC	TTATCTACAA	540
TTCGAGCTTG	CATGCCTGCA	GGTCGTTACA	TAACTTACGG	TAAATGGCCC	GCCTGGCTGA	600
CCGCCCAACG	ACCCCGCCCC	ATTGACGTCA	ATAATGACGT	ATGTTCCCAT	AGTAACGCCA	660
ATAGGGACTT	TCCATTGACG	TCAATGGGTG	GAGTATTTAC	GGTAAACTGC	CCACTTGGCA	720
GTACATCAAG	TGTATCATAT	GCCAAGTACG	CCCCCTATTG	ACGTCAATGA	CGGTAAATGG	780
CCGCCTGGC	ATTATGCCCA	GTACATGACC	TTATGGGACT	TTCCTACTTG	GCAGTACATC	840
TACGTATTAG	TCATCGCTAT	TACCATGGTG	ATGCGGTTTT	GGCAGTACAT	CAATGGGCGT	900
GGATAGCGGT	TTGACTCACG	GGGATTTCCA	AGTCTCCACC	CCATTGACGT	CAATGGGAGT	960
TTGTTTTGGC	ACCAAAATCA	ACGGGACTTT	CCAAAATGTC	GTAACAACTC	CGCCCCATTG	1020
ACGCAAATGG	GCGGTAGGCG	TGTACGGTGG	GAGGTCTATA	TAAGCAGAGC	TCGTTTAGTG	1080
AACCGTCAGA	TCGCCTGGAG	ACGCCATCCA	CGCTGTTTTG	ACCTCCATAG	AAGACACCGG	1140
GACCGATCCA	GCCTCCGGAC	TCTAGAGGAT	CCGGTACTCG	AGGAACTGAA	AAACCAGAAA	1200
GTTAACTGGT	AAGTTTAGTC	TTTTTGTCCT	TTATTTCAGG	TCCCGGATCC	GGTGGTGGTG	1260
CAAATCAAAG	AACTGCTCCT	CAGTGGATGT	TGCCTTTACT	TCTAGGCCTG	TACGGAAGTG	1320
TTACTTCTGC	TCTAAAAGCT	GCGGAATTGT	ACCCGCGGCC	GCAATTCCCG	GGGATCGAAA	1380
GAGCCTGCTA	AAGCAAAAAA	GAAGTCACCA	TGTCGTTTAC	TTTGACCAAC	AAGAACGTGA	1440
TTTTCGTTGC	CGGTCTGGGA	GGCATTGGTC	TGGACACCAG	CAAGGAGCTG	CTCAAGCGCG	1500
ATCCCGTCGT	TTTACAACGT	CGTGACTGGG	AAAACCCTGG	CGTTACCCAA	CTTAATCGCC	1560
TTGCAGCACA	TCCCCCTTTC	GCCAGCTGGC	GTAATAGCGA	AGAGGCCCGC	ACCGATCGCC	1620
CTTCCCAACA	GTTGCGCAGC	CTGAATGGCG	AATGGCGCTT	TGCCTGGTTT	CCGGCACCAG	1680
AAGCGGTGCC	GGAAAGCTGG	CTGGAGTGCG	ATCTTCCTGA	GGCCGATACT	GTCGTCGTCC	1740
TAATGTTTCT	GAAGCTGC	ACAGGAAAG	ACAGAGCCCG	AAATATTTCT	GATGGCGTTA	1800
ACTCGGCGTT	TCATCTGTGG	TGCAACGGGC	GCTGGGTGCG	TTACGGCCAG	GACAGTCGTT	1980

TGCCGTCTGA	ATTTGACCTG	AGCGCATT	TTT TACGCGCCGG	AGAAAACCGC	CTCGCGGTGA	2040
TGGTGCTGCG	TTGGAGTGAC	GGCAGTTATC	TGGAAGATCA	GGATATGTGG	CGGATGAGCG	2100
GCATTTTCCG	TGACGTCTCG	TTGCTGCATA	AACCGACTAC	ACAAATCAGC	GATTTCCATG	2160
TTGCCACTCG	CTTTAATGAT	GATTTTCAGCC	GCGCTGTACT	GGAGGCTGAA	GTTTCAGATGT	2220
GCGGCGAGTT	GCGTGACTAC	CTACGGGTAA	CAGTTTCTTT	ATGGCAGGGT	GAAACGCAGG	2280
TCGCCAGCGG	CACCGCGCCT	TTCGGCGGTG	AAATTATCGA	TGAGCGTGGT	GGTTATGCCG	2340
ATCGCGTCAC	ACTACGTCTG	AACGTCGAAA	ACCCGAAACT	GTGGAGCGCC	GAAATCCCGA	2400
ATCTCTATCG	TGCGGTGGTT	GAAGTGCACA	CCGCCGACGG	CACGCTGATT	GAAGCAGAAG	2460
CCTGCGATGT	CGGTTTCCGC	GAGGTGCGGA	TTGAAAATGG	TCTGCTGCTG	CTGAACGGCA	2520
AGCCGTTGCT	GATTCGAGGC	GTTAACCGTC	ACGAGCATCA	TCCTCTGCAT	GGTCAGGTCA	2580
TGGATGAGCA	GACGATGGTG	CAGGATATCC	TGCTGATGAA	GCAGAACAAC	TTTAACGCCG	2640
TGCGCTGTTT	GCATTATCCG	AACCATCCGC	TGTGGTACAC	GCTGTGCGAC	CGCTACGGCC	2700
TGTATGTGGT	GGATGAAGCC	AATATTGAAA	CCCACGGCAT	GGTGCCAATG	AATCGTCTGA	2760
CCGATGATCC	GCGCTGGCTA	CCGGCGATGA	GCGAACGCGT	AACGCGAATG	GTGCAGCGCG	2820
ATCGTAATCA	CCCGAGTGTG	ATCATCTGGT	CGCTGGGGAA	TGAATCAGGC	CACGGCGCTA	2880
ATCACGACGC	GCTGTATCGC	TGGATCAAAT	CTGTCGATCC	TTCCCGCCCCG	GTGCAGTATG	2940
AAGGCGGCGG	AGCCGACACC	ACGGCCACCG	ATATTATTTG	CCCGATGTAC	GCGCGCGTGG	3000
ATGAAGACCA	GCCCTTCCCG	GCTGTGCCGA	AATGGTCCAT	CAAAAATGG	CTTTCGCTAC	3060
CTGGAGAGAC	GCGCCCGCTG	ATCCTTTGCG	AATACGCCCA	CGCGATGGGT	AACAGTCTTG	3120
GCGGTTTCGC	TAAATACTGG	CAGGCGTTTC	GTCAGTATCC	CCGTTTACAG	GGCGGCTTCG	3180
TCTGGGACTG	GGTGGATCAG	TCGCTGATTA	AATATGATGA	AAACGGCAAC	CCGTGGTCCG	3240
CTTACGGCGG	TGATTTTGGC	GATACGCCGA	ACGATCGCCA	GTTCTGTATG	AACGGTCTGG	3300
TCTTTGCCGA	CCGCACGCCG	CATCCAGCGC	TGACGGAAGC	AAAACACCAG	CAGCAGTTTT	3360
TCCAGTTCCG	TTTATCCGGG	CAAACCATCG	AAGTGACCAG	CGAATACCTG	TTCCGTCATA	3420
GCGATAACGA	GCTCCTGCAC	TGGATGGTGG	CGCTGGATGG	TAAGCCGCTG	GCAAGCGGTG	3480
AAGTGCCTCT	GGATGTCGCT	CCACAAGGTA	AACAGTTGAT	TGAAGTGCCT	GAAGTACCGC	3540
AGCCGGAGAG	CGCCGGGCAA	CTCTGGCTCA	CAGTACGCGT	AGTGCAACCG	AACGCGACCG	3600
TTTGATCGA	TTGGTAAAT	AAGCGTTGG	AAATTAACCG	CAAGTCAAGG	TTTCTTTCA	
AGATGTGGAT	TGGCGATAAA	AAACAACCTG	TGACGCGGCT	GCGCGATCAG	TTCACCCGTG	3840

CACCGCTGGA	TAACGACATT	GGCGTAAGTG	AAGCGACCCG	CATTGACCCT	AACGCCTGGG	3900
TCGAACGCTG	GAAGGCGGCG	GGCCATTACC	AGGCCGAAGC	AGCGTTGTTG	CAGTGCACGG	3960
CAGATACACT	TGCTGATGCG	GTGCTGATTA	CGACCGCTCA	CGCGTGGCAG	CATCAGGGGA	4020
AAACCTTATT	TATCAGCCGG	AAAACCTACC	GGATTGATGG	TAGTGGTCAA	ATGGCGATTA	4080
CCGTTGATGT	TGAAGTGGCG	AGCGATACAC	CGCATCCGGC	GCGGATTGGC	CTGAAC TGCC	4140
AGCTGGCGCA	GGTAGCAGAG	CGGGTAAACT	GGCTCGGATT	AGGGCCGCAA	GAAAAC TATC	4200
CCGACCGCCT	TACTGCCGCC	TGTTTTGACC	GCTGGGATCT	GCCATTGTCA	GACATGTATA	4260
CCCCGTACGT	CTTCCCGAGC	GAAAACGGTC	TGCGCTGCGG	GACGCGCGAA	TTGAATTATG	4320
GGCCACACCA	GTGGCGCGGC	GACTTCCAGT	TCAACATCAG	CCGCTACAGT	CAACAGCAAC	4380
TGATGGAAAC	CAGCCATCGC	CATCTGCTGC	ACGCGGAAGA	AGGCACATGG	CTGAATATCG	4440
ACGGTTTCCA	TATGGGGATT	GGTGGCGACG	ACTCCTGGAG	CCCGTCAGTA	TCGGCGGAAT	4500
TACAGCTGAG	CGCCGGTCGC	TACCATTACC	AGTTGGTCTG	GTGTCAAAAA	TAATAATAAC	4560
CGGGCAGGCC	ATGTCTGCCC	GTATTTGCGG	TAAGGAAATC	CATTATGTAC	TATTTAAAAA	4620
ACACAAACTT	TTGGATGTTT	GGTTTATTCT	TTTTCTTTTA	CTTTTTTATC	ATGGGAGCCT	4680
ACTTCCCGTT	TTTCCCGATT	TGGCTACATG	ACATCAACCA	TATCAGCAAA	AGTGATACGG	4740
GTATTATTTT	TGCCGCTATT	TCTCTGTTCT	CGCTATTATT	CCAACCGCTG	TTTGGTCTGC	4800
TTTCTGACAA	ACTCGGCCTC	GACTCTAGGC	GGCCGCGGGG	ATCCAGACAT	GATAAGATAC	4860
ATTGATGAGT	TTGGACAAAC	CACAACTAGA	ATGCAGTGAA	AAAAATGCTT	TATTTGTGAA	4920
ATTTGTGATG	CTATTGCTTT	ATTTGTAACC	ATTATAAGCT	GCAATAAACA	AGTTAACAAC	4980
AACAATTGCA	TTCATTTTAT	GTTTCAGGTT	CAGGGGGAGG	TGTGGGAGGT	TTTTTCGGAT	5040
CCTCTAGAGT	CGAGTAGATA	AGTAGCATGG	CGGGTTAATC	ATTAAC TACA	AGGAACCCCT	5100
AGTGATGGAG	TTGGCCACTC	CCTCTCTGCG	CGCTCGCTCG	CTCACTGAGG	CCGGGCGACC	5160
AAAGGTCGCC	CGACGCCCCG	GCTTTGCCCC	GGCGGCCTCA	GTGAGCGAGC	GAGCGCGCAG	5220
CAGATCTGGA	AGGTGCTGAG	GTACGATGAG	ACCCGCACCA	GGTG CAGACC	CTGCGAGTGT	5280
GGCGGTAAAC	ATATTAGGAA	CCAGCCTGTG	ATGCTGGATG	TGACCGAGGA	GCTGAGGCCC	5340
GATCACTTGG	TGCTGGCCTG	CACCCGCGCT	GAGTTTGGCT	CTAGCGATGA	AGATACAGAT	5400
TGAGGTACTG	AAATGTGTGG	GCGTGGCTTA	AGGGTGGGAA	AGAATATATA	AGGTGGGGGT	5460
AGAAATGTA	TGGGTCGAG	CATTGATGG	CGCCCGCTCA	TGACCGCAAA	CTCTACTACC	5520
TTGACCTACG	AGACCGTGTC	TGGAACGCCG	TTGGAGACTG	CAGCCTCCGC	CGCCGCTTCA	5700

GCCGCTGCAG	CCACCGCCCG	CGGGATTGTG	ACTGACTTTG	CTTTCCTGAG	CCCGCTTGCA	5760
AGCAGTGCAG	CTTCCCGTTC	ATCCGCCCCG	GATGACAAGT	TGACGGCTCT	TTTGGCACAA	5820
TTGGATTCTT	TGACCCGGGA	ACTTAATGTC	GTTTCTCAGC	AGCTGTTGGA	TCTGCGCCAG	5880
CAGGTTTCTG	CCCTGAAGGC	TTCCTCCCCT	CCCAATGCCG	TTTAAACAT	AAATAAAAAA	5940
CCAGACTCTG	TTTGGATTG	GATCAAGCAA	GTGTCTTGCT	GTCTTTATTT	AGGGGTTTTG	6000
CGCGCGCGGT	AGGCCCCGGA	CCAGCGGTCT	CGGTCGTTGA	GGGTCTGTG	TATTTTTTCC	6060
AGGACGTGGT	AAAGGTGACT	CTGGATGTTT	AGATACATGG	GCATAAGCCC	GTCTCTGGGG	6120
TGGAGGTAGC	ACCACTGCAG	AGCTTCATGC	TGCGGGGTGG	TGTTGTAGAT	GATCCAGTCG	6180
TAGCAGGAGC	GCTGGGCGTG	GTGCCTAAAA	ATGCTTTTCA	GTAGCAAGCT	GATTGCCAGG	6240
GGCAGGCCCT	TGGTGTAAGT	GTTTACAAAG	CGGTTAAGCT	GGGATGGGTG	CATACGTGGG	6300
GATATGAGAT	GCATCTTGGA	CTGTATTTTT	AGGTTGGCTA	TGTTCCAGC	CATATCCCTC	6360
CGGGGATTCA	TGTTGTGCAG	AACCACCAGC	ACAGTGATATC	CGGTGCACTT	GGGAAATTTG	6420
TCATGTAGCT	TAGAAGGAAA	TGCGTGGAAG	AACCTGGAGA	CGCCCTTGTC	ACCTCCAAGA	6480
TTTTCCATGC	ATTCGTCCAT	AATGATGGCA	ATGGGCCAC	GGGCGGCGGC	CTGGGCGAAG	6540
ATATTTCTGG	GATCACTAAC	GTCATAGTTG	TGTTCCAGGA	TGAGATCGTC	ATAGGCCATT	6600
TTTACAAAGC	GCGGGCGGAG	GGTGCCAGAC	TGCGGTATAA	TGGTTCCATC	CGGCCCAGGG	6660
GCGTAGTTAC	CCTCACAGAT	TTGCATTTCC	CACGCTTTGA	GTTCAGATGG	GGGGATCATG	6720
TCTACCTGCG	GGGCGATGAA	GAAAACGGTT	TCCGGGGTAG	GGGAGATCAG	CTGGGAAGAA	6780
AGCAGGTTCC	TGAGCAGCTG	CGACTTACCG	CAGCCGGTGG	GCCCCTAAAT	CACACCTATT	6840
ACCGGGTGCA	ACTGGTAGTT	AAGAGAGCTG	CAGCTGCCGT	CATCCCTGAG	CAGGGGGGCC	6900
ACTTCGTTAA	GCATGTCCCT	GACTCGCATG	TTTTCCCTGA	CCAAATCCGC	CAGAAGGCGC	6960
TCGCCGCCCA	GCGATAGCAG	TTCTTGCAAG	GAAGCAAAGT	TTTTCAACGG	TTTGAGACCG	7020
TCCGCCGTAG	GCATGCTTTT	GAGCGTTTGA	CCAAGCAGTT	CCAGGCGGTC	CCACAGCTCG	7080
GTCACCTGCT	CTACGGCATC	TCGATCCAGC	ATATCTCCTC	GTTTCGCGGG	TTGGGGCGGC	7140
TTTCGCTGTA	CGGCAGTAGT	CGGTGCTCGT	CCAGACGGGC	CAGGGTCATG	TCTTTCCACG	7200
GGCGCAGGGT	CCTCGTCAGC	GTAGTCTGGG	TCACGGTGAA	GGGGTGCGCT	CCGGGCTGCG	7260
CGCTGGCCAG	GGTGCGCTTG	AGGCTGGTCC	TGCTGGTGCT	GAAGCGCTGC	CGGTCTTCGC	7320
AGGCCCCGCA	GACGGTCTCG	CATTCCACGA	GCCAGGTGAG	CTCTGGCCGT	TCGGGGTCAA	7560

AAACCAGGTT	TCCCCATGC	TTTTTGATGC	GTTTCTTACC	TCTGGTTTCC	ATGAGCCGGT	7620
GTCCACGCTC	GGTGACGAAA	AGGCTGTCCG	TGTCCCGTA	TACAGACTTG	AGAGGCCTGT	7680
CCTCGACCGA	TGCCCTTGAG	AGCCTTCAAC	CCAGTCAGCT	CCTTCCGGTG	GGCGCGGGGC	7740
ATGACTATCG	TCGCCGCACT	TATGACTGTC	TTCTTTATCA	TGCAACTCGT	AGGACAGGTG	7800
CCGGCAGCGC	TCTGGGTCAT	TTTCGGCGAG	GACCGCTTTC	GCTGGAGCGC	GACGATGATC	7860
GGCCTGTGCG	TTGCGGTATT	CGGAATCTTG	CACGCCCTCG	CTCAAGCCTT	CGTCACTGGT	7920
CCCGCCACCA	AACGTTTCGG	CGAGAAGCAG	GCCATTATCG	CCGGCATGGC	GGCCGACCGG	7980
CTGGGCTACG	TCTTGCTGGC	GTTTCGCGACG	CGAGGCTGGA	TGGCCTTCCC	CATTATGATT	8040
CTTCTCGCTT	CCGGCGGCAT	CGGGATGCCC	GCGTTGCAGG	CCATGCTGTC	CAGGCAGGTA	8100
GATGACGACC	ATCAGGGACA	GCTTCAAGGA	TCGCTCGCGG	CTCTTACCAG	CCTAACTTCG	8160
ATCACTGGAC	CGCTGATCGT	CACGGCGATT	TATGCCGCCT	CGGCGAGCAC	ATGGAACGGG	8220
TTGGCATGGA	TTGTAGGCGC	CGCCCTATAC	CTTGCTGCGC	TCCCCGCGTT	GCGTCGCGGT	8280
GCATGGAGCC	GGGCCACCTC	GACCTGAATG	GAAGCCGGCG	GCACCTCGCT	AACGGATTCA	8340
CCACTCCAAG	AATTGGAGCC	AATCAATTCT	TGCGGAGAAC	TGTGAATGCG	CAAACCAACC	8400
CTTGCGAGAA	CATATCCATC	GCGTCCGCCA	TCTCCAGCAG	CCGCACGCGG	CGCATCTCGG	8460
GCAGCGTTGG	GTCCTGGCCA	CGGGTGCGCA	TGATCGTGCT	CCTGTCGTTG	AGGACCCGGC	8520
TAGGCTGGCG	GGGTGCGCTT	ACTGGTTAGC	AGAATGAATC	ACCGATACGC	GAGCGAACGT	8580
GAAGCGACTG	CTGCTGCAAA	ACGTCTGCGA	CCTGAGCAAC	AACATGAATG	GTCTTCGGTT	8640
TCCGTGTTTC	GTAAAGTCTG	GAAACGCGGA	AGTCAGCGCC	CTGCACCATT	ATGTTCCGGA	8700
TCTGCATCGC	AGGATGCTGC	TGGCTACCCT	GTGGAACACC	TACATCTGTA	TTAACGAAGC	8760
CTTTCTCAAT	GCTCACGCTG	TAGGTATCTC	AGTTCGGTGT	AGGTCGTTTC	CTCCAAGCTG	8820
GGCTGTGTGC	ACGAACCCCC	CGTTCAGCCC	GACCGCTGCG	CCTTATCCGG	TAATCTATCGT	8880
CTTGAGTCCA	ACCCGGTAAG	ACACGACTTA	TCGCCACTGG	CAGCAGCCAC	TGGTAACAGG	8940
ATTAGCAGAG	CGAGGTATGT	AGGCGGTGCT	ACAGAGTTCT	TGAAGTGGTG	GCCTAACTAC	9000
GGCTACACTA	GAAGGACAGT	ATTGGGTATC	TGCGCTCTGC	TGAAGCCAGT	TACCTTCGGA	9060
AAAAGAGTTG	GTAGCTCTTG	ATCCGGCAAA	CAAACACCG	CTGGTAGCGG	TGGTTTTTTT	9120
GTTTGCAAGC	AGCAGATTAC	GCGCAGAAAA	AAAGGATCTC	AAGAAGATCC	TTTGATCTTT	9180
TAAGTATAA	ATGAGTAAAC	TTGGTCTGAC	AGTTACCAA	GCTTAATCAG	TGAGGCACCT	9240
ATCTCAGCGA	TCTGTCTATT	TCGTTTATCC	ATAGTTGCCT	GAATCCCCGT	CGTGTAGATA	9420



31

ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG CAATGATACC GCGAGACCCA	9480
CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAGCCAG CCGGAAGGGC CGAGCGCAGA	9540
AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA	9600
GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTGC AGGCATCGTG	9660
GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG ATCAAGGCGA	9720
GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC TCCGATCGTT	9780
GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA TGGCAGCACT GCATAATTCT	9840
CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA	9900
TTCTGAGAAT AGTGATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAC ACGGGATAAT	9960
ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTT TTCGGGGCGA	10020
AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA TGTAAACCCAC TCGTGCACCC	10080
AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG GGTGAGCAAA AACAGGAAGG	10140
CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC	10200
CTTTTTCAAT ATTATTGAAG CATTATCAG GGTATTGTC TCATGAGCGG ATACATATTT	10260
GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA CATTTCCCCG AAAAGTGCCA	10320
CCTGACGTCT AAGAAACCAT TATTATCATG ACATTAACCT ATAAAAATAG GCGTATCACG	10380
AGGCCCTTTC GTCTTCAA	10398

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGCCTCTAGA TGGAGGGGTG GAGTCGTGAC	30
----------------------------------	----

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGCCGGATCC AACGCGCAGC CGCCATGCCG	30
----------------------------------	----

32

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGCCGGATCC CAAACCTCCC GCTTCAAAT

30

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCCGAGCTC AGGCTGGGTT TTGGGGAGCA

30

## WHAT IS CLAIMED IS:

1. A method for production of recombinant adeno-associated virus (AAV) comprising culturing a host cell comprising and capable of expressing
  - (a) a cre transgene, which permits splicing out of the rep and cap gene inhibitory sequences that when removed lead to activation of rep and cap;
  - (b) AAV rep and cap genes having a spacer 5' thereto, said spacer flanked by lox sites;
  - (c) a minigene comprising a therapeutic transgene flanked by AAV inverse terminal repeats (ITRs);in the presence of sufficient helper virus functions, wherein a recombinant AAV capable of expressing said transgene is produced.
2. The method according to claim 1 further comprising:
  - (a) introducing into a selected host cell
    - i. a first vector comprising a cre gene under control of sequences which permit expression of cre recombinase;
    - ii. a second vector comprising from 5' to 3', a selected promoter, a spacer sequence flanked by loxP sites, an AAV rep gene and an AAV cap gene;
    - iii. a third vector comprising a minigene consisting essentially of, from 5' to 3', a 5' AAV inverse terminal repeat (ITR), a selected promoter, a selected transgene and 3' AAV ITR;
  - (b) culturing the host cell underexpressing the product of said transgene.

3. The method according to claim 1 wherein at least one of said vectors is a recombinant adenovirus and the host cell is a 293 cell.

4. The method according to claim 1 wherein the first vector is a recombinant adenovirus and the sequences which permit expression comprise a cytomegalovirus promoter, the vector further comprising a nuclear localization signal operably linked to the cre gene.

5. The method according to claim 1 wherein the second vector is a recombinant adenovirus and the selected promoter comprises AAV P5.

6. The method according to claim 5 wherein the spacer sequence is selected from the group consisting of:

- (a) a 1300 bp fragment containing translational start and stop sequences;
- (b) a 1600 bp fragment containing the GFP cDNA, an intron and a polyadenylation signal; and
- (c) a 1000 bp fragment containing the neomycin coding sequence and a polyadenylation signal.

7. A method for production of recombinant adeno-associated virus (AAV) comprising:

- (a) providing a host cell expressing cre;
- (b) introducing into said host cell a first vector comprising from 5' to

a second vector comprising from 5' to 3', a minigene consisting essentially of 5' AAV inverse terminal repeat (ITR), a selected promoter, a selected transgene, and a 3' AAV ITR;

(c) culturing the host cell under conditions which permit expression of the cre recombinase and replication and packaging of a recombinant AAV; and

(d) recovering the recombinant AAV capable of expressing the product of the transgene.

8. The method according to claim 7 wherein the first and second vectors are recombinant adenoviruses.

9. The method according to claim 8 wherein the spacer sequence is selected from the group consisting of:

(a) a 1300 bp fragment containing translational start and stop sequences;

(b) a 1600 bp fragment containing the GFP cDNA, an intron and a polyadenylation signal; and

(c) a 1000 bp fragment containing the neomycin coding sequence and a polyadenylation signal.

10. A recombinant AAV produced according to the method of any one of claims 1 - 9.

1/8

## GFP SPACER

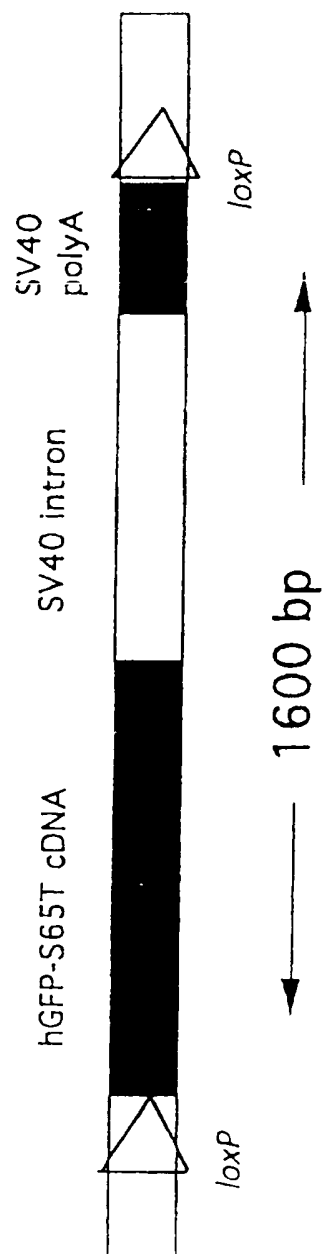


Fig. 1

## NEO SPACER

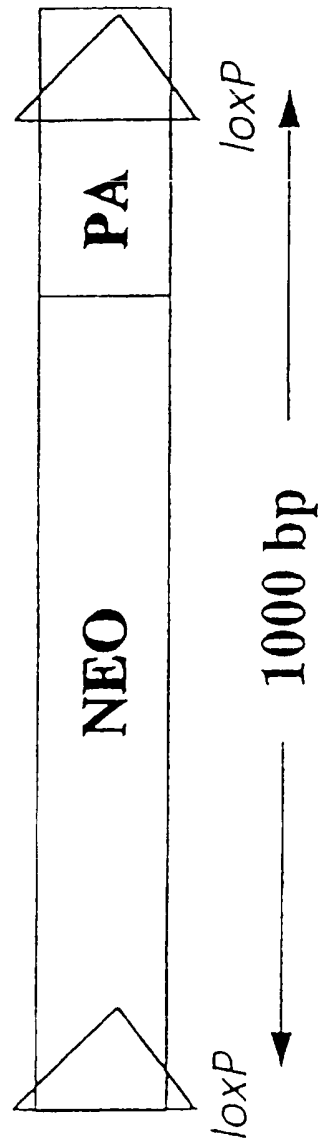


Fig. 2

3/8

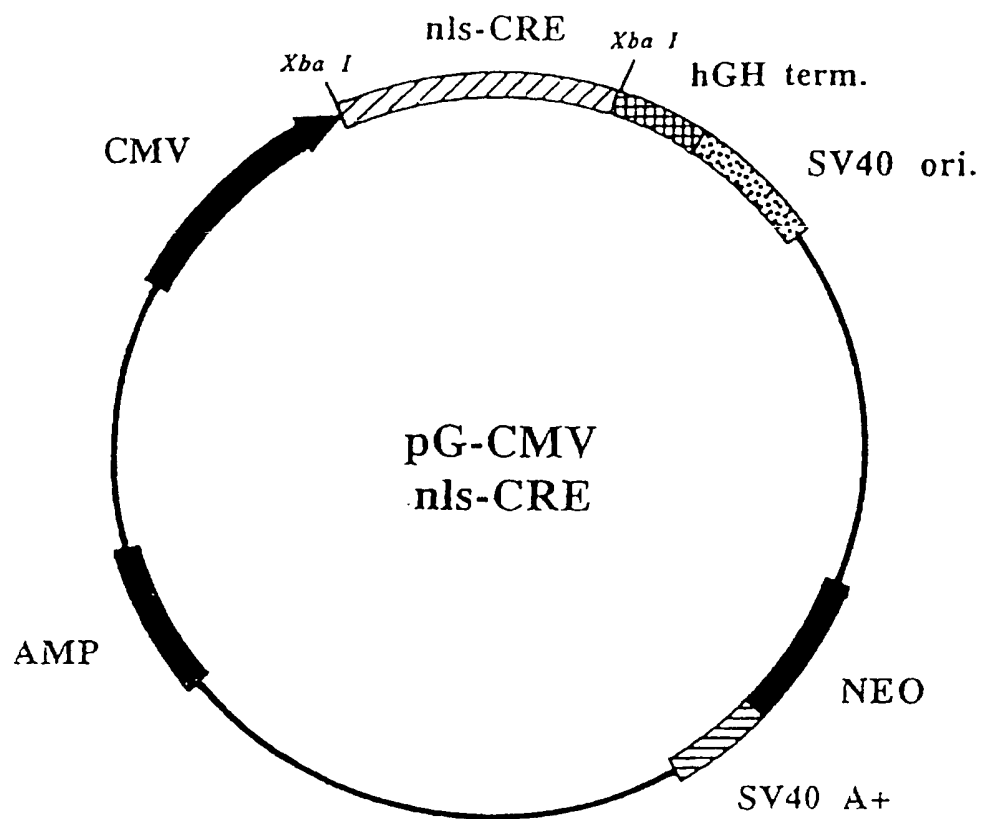
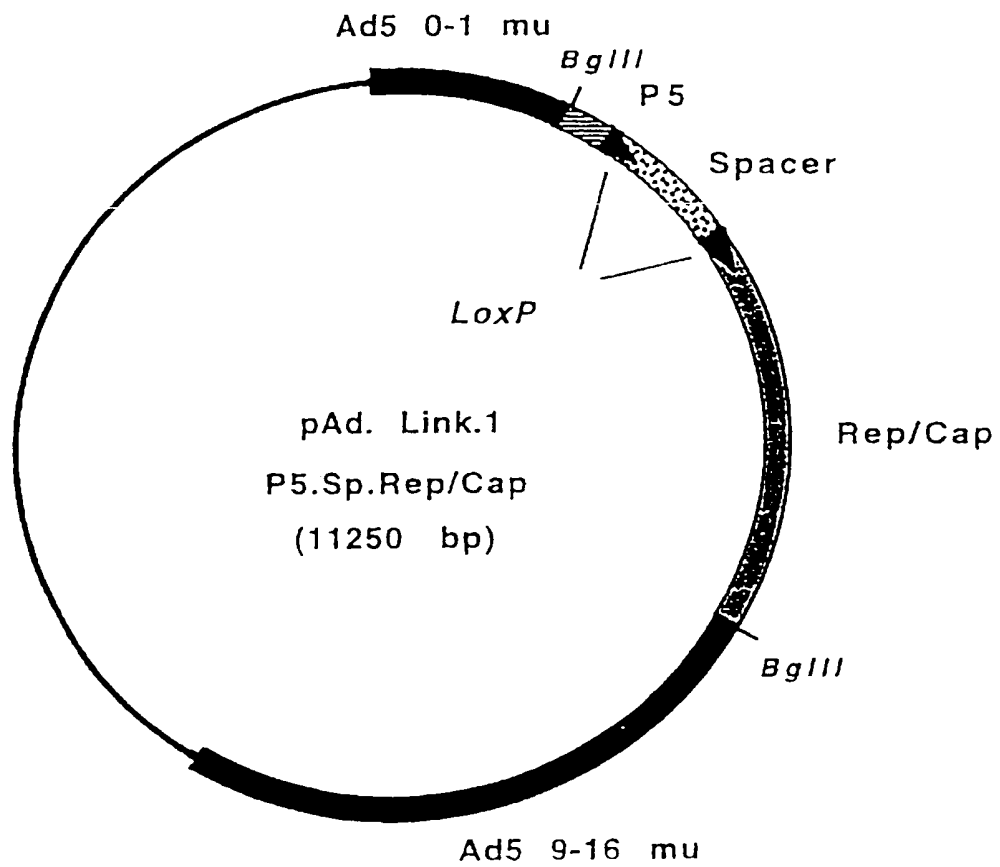
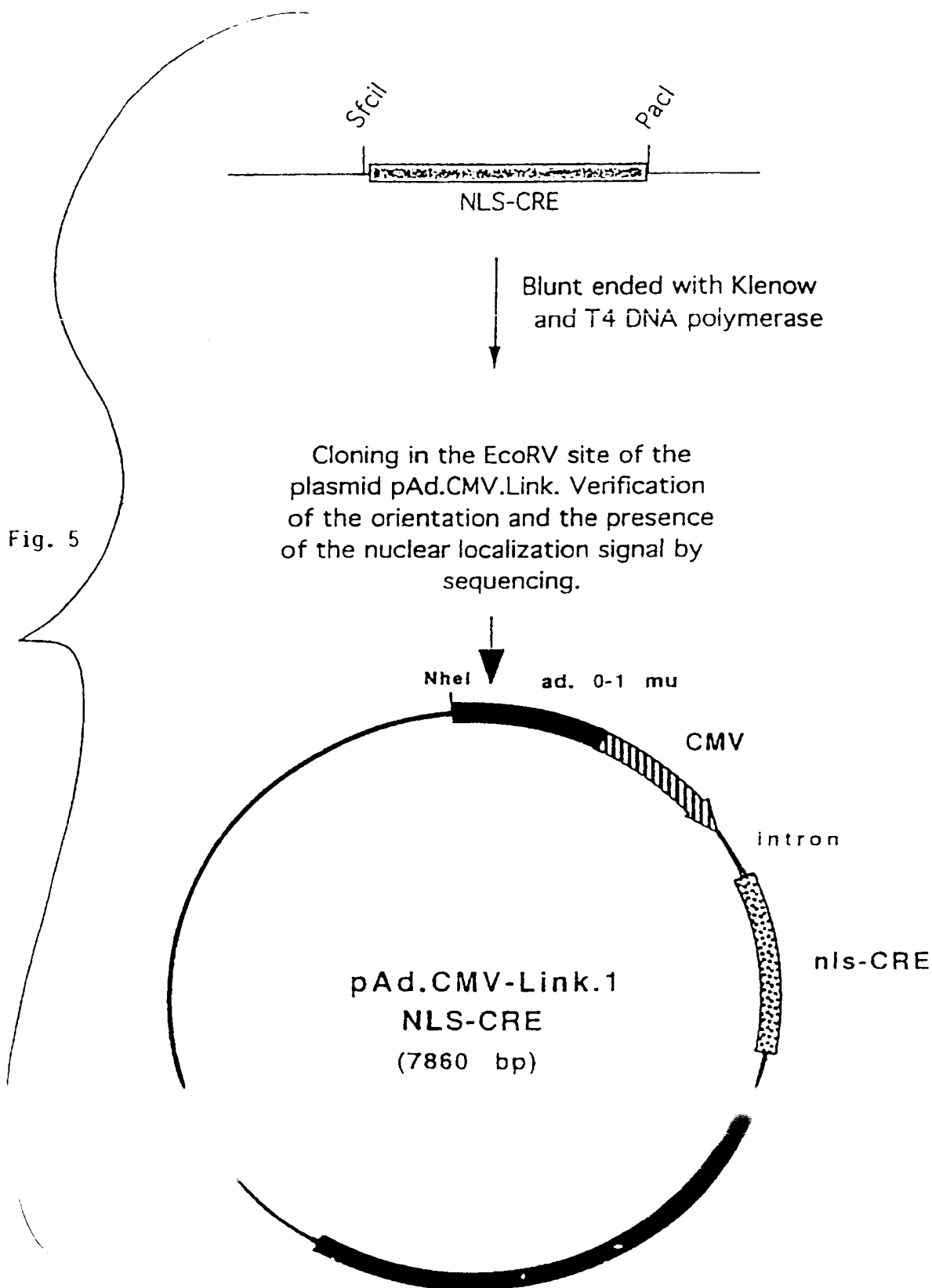


Fig. 3



4/8



Construction of the Ad. CMV. NLS-CRE

6/8

Fig. 6A

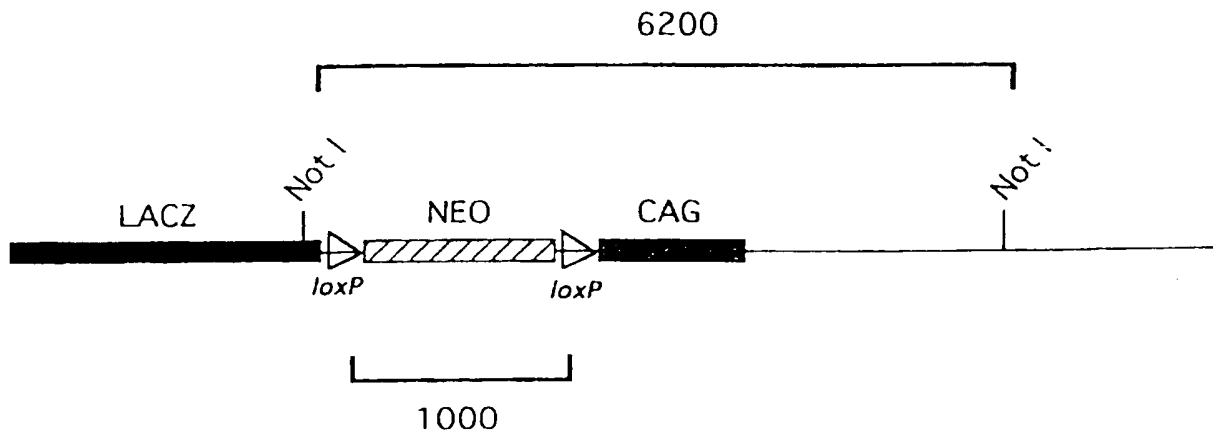




Fig. 6B

Fig. 6C

Fig. 6D

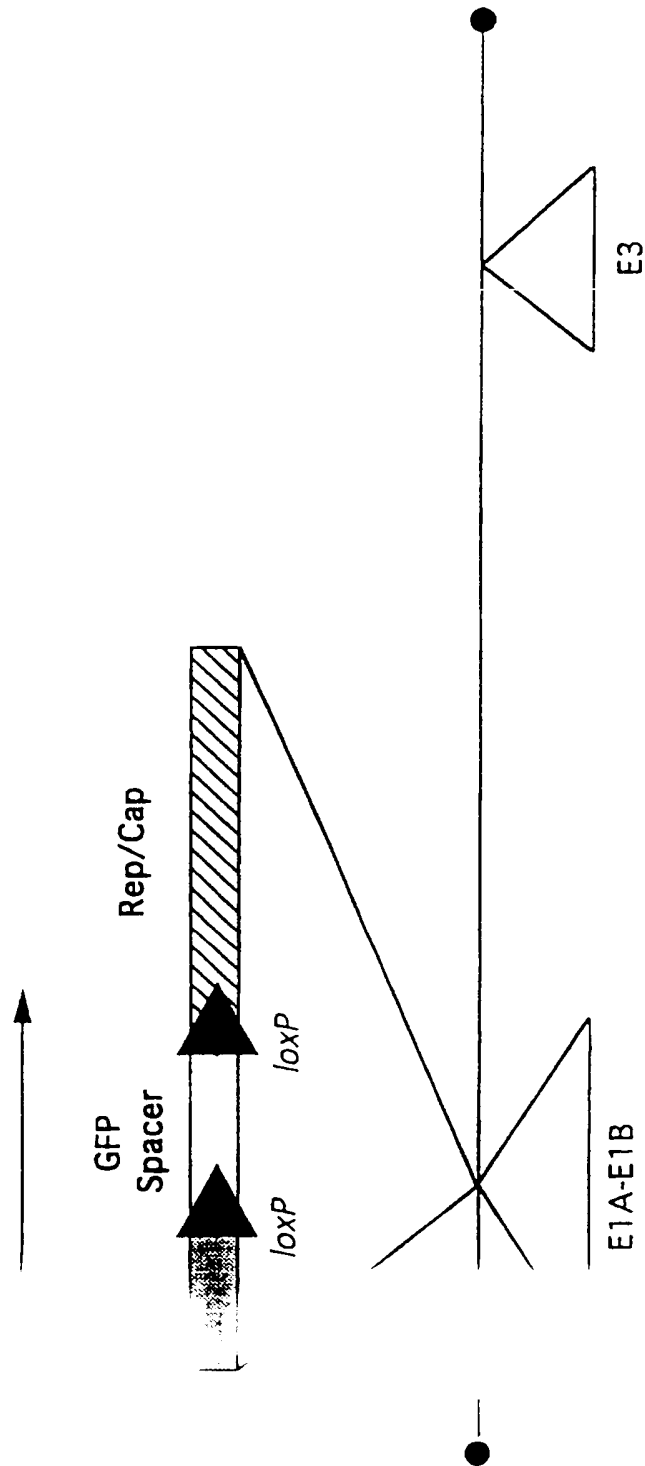


FIG. 7

## A CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C07K14/015 C12N9/00 C12N9/52 C12N15/35  
C12N7/01

According to international Patent Classification (IPC) or to both national classification and IPC

## B FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 17947 A (TARGETED GENETICS CORP ; ALLEN JAMES M (US)) 13 June 1996 see the whole document	1-10
Y	ANTON M ET AL: "SITE-SPECIFIC RECOMBINATION MEDIATED BY AN ADENOVIRUS VECTOR EXPRESSING THE CRE RECOMBINASE PROTEIN: A MOLECULAR SWITCH FOR CONTROL OF GENE EXPRESSION" JOURNAL OF VIROLOGY, vol. 69, no. 8, August 1995, pages 4600-4606, XP002011775 see page 4602, column 1, line 4 - page 4604, column 2, line 41; figures 1.4A, 5A --- -/--	1-10

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex

## Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referred to in the description of the invention

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is taken alone

13 February 1998

23/02/1998

Name and mailing address of the ISA

Authorized signatory

International Patent  
Classification  
C12N15/86  
C07K14/015  
C12N9/00  
C12N9/52  
C12N15/35  
C12N7/01

Name of the ISA

## C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
Y	KANEGAE Y ET AL: "EFFICIENT GENE ACTIVATION IN MAMMALIAN CELLS BY USING RECOMBINANT ADENOVIRUS EXPRESSING SITE-SPECIFIC CRE RECOMBINASE" NUCLEIC ACIDS RESEARCH, vol. 23, no. 19, 11 October 1995, pages 3816-3821, XP002011774 see page 3818, column 1, paragraph 4 - page 3819, column 1, paragraph 2; figures 1,3 see page 3821, column 1, paragraph 5	1-3,6-10
Y	WANG P ET AL: "HIGH FREQUENCY RECOMBINATION BETWEEN LOXP SITES IN HUMAN CHROMOSOMES MEDIATED BY AN ADENOVIRUS VECTOR EXPRESSING CRE RECOMBINASE" SOMATIC CELL AND MOLECULAR GENETICS, vol. 21, no. 6, November 1995, pages 429-441, XP000617918 see page 439, column 2, paragraph 2 - page 440, column 1, paragraph 2	1-4
P,X	KANEGAE Y ET AL: "Efficient gene activation system on mammalian cell chromosomes using recombinant adenovirus producing Cre recombinase" GENE, vol. 181, no. 1-2, 28 November 1996, page 207-212 XP004071882 see page 212, column 1, line 1 - line 10; figure 1	1-10
Y	WO 95 13392 A (OHIO MED COLLEGE ;TARGETED GENETICS CORP (US); TREMPER JAMES P (US)) 18 May 1995 see the whole document	1-10
Y	WO 95 13365 A (TARGETED GENETICS CORP ;UNIV JOHNS HOPKINS (US); FLOTTE TERENCE R) 18 May 1995 see the whole document	1-10
Y,P	WO 97 06272 A (AVIGEN INC) 20 February 1997 see page 6, line 28 - page 7, line 4	1-5,7,8, 10
A	SNAITH M R ET AL: "Multiple cloning sites" NUCLEIC ACIDS RESEARCH, vol. 166, no. 1, 1 January 1995, page 173-174 XP004043130	1

## C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document with indication where appropriate of the relevant passages	Relevant to claim No
A	BEATON A ET AL: "EXPRESSION FROM THE ADENO-ASSOCIATED VIRUS P5 AND P19 PROMOTERS IS NEGATIVELY REGULATED IN TRANS BY THE REP PROTEIN" JOURNAL OF VIROLOGY, vol. 63, no. 10, October 1989, pages 4450-4454, XP000609489 -----	1



Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9617947 A	13-06-96	AU 4596396 A	26-06-96
		CA 2207927 A	13-06-96
		EP 0796339 A	24-09-97
WO 9513392 A	18-05-95	AU 678867 B	12-06-97
		AU 8130994 A	29-05-95
		CA 2176215 A	18-05-95
		EP 0728214 A	28-08-96
		JP 9510602 T	28-10-97
WO 9513365 A	18-05-95	AU 1129395 A	29-05-95
		CA 2176117 A	18-05-95
		EP 0733103 A	25-09-96
		JP 9509564 T	30-09-97
		US 5658776 A	19-08-97
WO 9706272 A	20-02-97	US 5622856 A	22-04-97